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Research report

Cerebellar CB_1 receptor mediation of Δ^9-THC-induced motor incoordination and its potentiation by ethanol and modulation by the cerebellar adenosinergic A_1 receptor in the mouse

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Abstract

The effect of intracerebellar microinfusion of antisense oligodeoxynucleotide to Δ^9-tetrahydrocannabinol (Δ^9-THC) and other naturally occurring cannabinoid receptor (CB_1) mRNA on Δ^9-THC-induced motor impairment was investigated in mice. Δ^9-THC (15–30 μg/μl; intracerebellar) resulted in a significant motor impairment in a dose-related manner. The intracerebellar pretreatment with antisense oligodeoxynucleotide (3.0 μg/100 nl/12 h; six administrations/mouse) virtually abolished Δ^9-THC (15 and 25 μg/1 μl; intracerebellar)-induced motor impairment. However, intracerebellar pretreatment with the mismatched oligodeoxynucleotide in exactly the same manner as the antisense was completely ineffective in altering the Δ^9-THC-induced motor impairment. These results strongly suggest the involvement of CB_1 receptor in the expression of Δ^9-THC-induced motor impairment. The intracerebellar microinfusion of adenosine A_1-selective agonist, N^6-cyclohexyladenosine (CHA) (4 ng/100 nl) significantly enhanced Δ^9-THC-induced motor impairment, suggesting a cerebellar A_1 adenosinergic modulation of motor impairment. A pretreatment with the antisense and the mismatched oligodeoxynucleotide also markedly attenuated and did not alter, respectively, the cerebellar A_1 adenosinergic modulation (enhancement) of Δ^9-THC-induced motor impairment. There was no change in the normal motor coordination due to intracerebellar pretreatment with antisense and its mismatch, in the presence as well as absence of intracerebellar CHA indicating the selectivity of interactions with Δ^9-THC. The Δ^9-THC-induced motor incoordination was also significantly enhanced dose-dependently by systemic (i.p.) ethanol administration suggesting behavioral synergism between the two psychoactive drugs. Pretreatment (intracerebellar) with pertussis toxin (PTX) markedly attenuated Δ^9-THC- and Δ^9-THC+CHA-induced motor incoordination suggesting coupling of CB_1 receptor to PTX-sensitive G-protein (G_i/G_o). These data suggested co-modulation by cerebellar cannabinoid and adenosine system of Δ^9-THC-induced motor impairment. Conversely, the results in the present study also suggested co-modulation by cerebellar adenosine A_1 and CB_1 receptors of ethanol-induced motor impairment, thereby indicating a possible common signal transduction pathway in the expression of motor impairment produced by Δ^9-THC as well as ethanol. © 2000 Elsevier Science B.V. All rights reserved.

Themes: Neural basis of behavior

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1. Introduction

Motor impairment is one of the well recognized effect of the marijuana consumption in humans. Δ^9-Tetrahydrocannabinol (Δ^9-THC), the main psychoactive component in marijuana [21] as well as in other naturally occurring and synthetic cannabinoids, has been well known to produce motor impairment [11,14] in humans as well as in laboratory animals. Motor impairment by cannabinoids has been suggested to be mediated by specific CB_1 [15,20] receptor located in the brain. This (CB_1) subtype of the cannabinoid receptor is the only receptor known to be present within the mammalian brain [12]. Presence of high densities of CB_1 have been shown by radioligand binding studies in the brain areas associated with memory, pain,

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movement control and motor functions [1,16]. The presence of high density of cannabinoid receptors on the axons and terminals of the GABAergic striatal neurons of the basal ganglia and of the glutamatergic granule cells of the cerebellum suggests a modulatory role in the control of movements and motor functions [15,20].

Cannabinoids have been shown to exert direct cellular effects on a number of organs, including the immune system, the liver and reproductive systems. Most of the behavioral and pharmacological effects so far studied appear to involve CB_1 receptor subtype. However, in spite of the important discovery of endogenous cannabimimetic ligands, anandamide [10] and more recently, 2-arachidonylglycerol [17,23], further studies may provide greater insight into the physiological and pathophysiological role of cannabinoid receptor system. The involvement of CB_1 receptor has been suggested in the expression of pharmacological effects such as alterations in locomotor activity, cognition, memory as well as in analgesia and appetite stimulation [15,20]. The possibility of another unidentified subytype of cannabinoid receptor has been raised by Pertwee et al. [20]. The present investigation was undertaken to delineate the role of cerebellar CB_1 subtype of the cannabinoid receptor in the motor impairment produced by Δ^9-THC. To demonstrate that the well-known motor impairment effects of cannabinoids are expressed due to participation of cerebellar CB_1 receptors, we have utilized the novel approach using antisense oligodeoxynucleotides in the present study. An antisense oligodeoxynucleotide is a short component of synthetic DNA having a nucleotide sequence that is the reverse of, and complementary to, a part of mRNA. Consequently, it binds to mRNA thereby preventing the translation of mRNA and inhibits the protein synthesis. The antisense oligodeoxynucleotides against receptors of neurotransmitters/neuromodulators have been synthesized and successfully used as a tool to investigate the functional role of these receptor systems by a reduction in the receptor number and functions [24]. Recently, Edsall et al. [12] have demonstrated that i.c.v. administration of antisense oligodeoxynucleotide to brain CB_1 cannabinoid receptor inhibited the antinociception produced by i.c.v.-injected cannabinoid agonist CP-55 940. Therefore, the effect of direct intracerebellar microinfusion of antisense oligodeoxynucleotide to CB_1 receptor was evaluated on the: (i) motor incoordination produced by intracerebellar Δ^9-THC and (ii) on the accentuation by intracerebellar adenosine agonist of motor impairment produced by Δ^9-THC in the mice.

Motor impairment by Δ^9-THC was used as the test response as evaluated by rotorod procedure. Although rotorod is not a selective procedure, we have been successfully using it with proper controls for more than a decade [2–5,8,9]. We report in this investigation that cerebellar adenosine modulates Δ^9-THC-induced motor impairment. It was then thought logical to investigate if the adenosinergic modulation involved an interaction with CB_1 receptors. Using the same approach i.e., the intracerebellar microinfusion of antisense oligodeoxynucleotide to CB_1 receptor 'knock down', we observed that the cerebellar CB_1 receptor indeed participates in the expression of intracerebellar Δ^9-THC-induced motor impairment as well as in the observed behavioral interactions between adenosine and Δ^9-THC. Both ethanol and cannabinoids are known to cause motor impairment in humans as well as in laboratory animals. Also, adenosine A_1 and CB_1 receptors are co-localized on the axons and axonal terminals of the cerebellar granule cells. We have previously demonstrated [2–5,8,9] brain A_1 adenosinergic modulation of ethanol-induced motor impairment. In view of this background, strong circumstantial evidence is present for possible synergism of motor impairment produced by ethanol and Δ^9-THC. Therefore, the possibility of behavioral interactions between these two psychoactive substances was also investigated.

2. Materials and methods

2.1. Synthesis of oligodeoxynucleotide

The synthesis of the antisense oligodeoxynucleotide was carried out by the Nucleic Acid Core Facility of the Lineberger Comprehensive Cancer Center using Applied Biosystems at the University of North Carolina (Chapel Hill, NC, USA). The sequences of the antisense and the mismatched nucleotide used in the present study were the same as reported by Edsall et al. [12] and are as follows: 5′-TCCGTCTAAGATCGACTT-3′ (antisense) and 5′-ACCGGCTATTATCTACTG-3′ (mismatch). The antisense oligodeoxynucleotide used was complementary to the 5′ end of the coding sequence in mouse CB_1 mRNA. Antisense and mismatch oligodeoxynucleotides were dissolved in sterile deionized water to give a final concentration of 3 μg/100 nl. The oligodeoxynucleotides were microinfused directly into the cerebellum (3 μg per intracerebellar injection) twice daily (0830 h and 2030 h) for 3 days. Eight groups of three mice each were used for the antisense experiments. Four groups were microinfused with the antisense oligodeoxynucleotide for 3 days, followed 12 h after the last administration by Δ^9-THC (15 μg) to two groups and both N^6-cyclohexyladenosine (CHA) (4 ng) and Δ^9-THC (15 μg) to other two groups by intracerebellar microinfusion. Similarly, four groups were treated with the mismatch oligodeoxynucleotide for 3 days, followed 12 h after the last administration by the intracerebellar microinfusion of same dose of Δ^9-THC to two groups and CHA and Δ^9-THC to the remaining two groups. The additional details about the antisense and the mismatch nucleotides were as reported by Edsall et al. [10] The antisense and sense nucleotides used in the present investigation were in phosphorothioate form.

2.2. Animals

Only male CD-1 mice of 25–28 g body weight and 5–6 weeks old were used in this study. These were purchased from Charles River Laboratories (Raleigh, NC, USA). Following implantation of the permanent indwelling stainless steel guide cannulas into the cerebellar cortex by stereotaxic surgery, the animals were housed individually. In the housing facility the temperature and the relative humidity were controlled within 23–25°C and 60–80%, respectively, with a 12 h light–dark cycle (lights on 0800–2000 h). Commercial pellet food and tap water were available ad lib except during the actual experimentation.

2.3. Surgery

Mice anesthetized with chloral hydrate (450 mg/kg, i.p.) were subjected to stereotaxic surgery under aseptic conditions in order to implant permanent stainless steel guide cannulas (22-gauge) into cerebellar cortex for drug microinfusions. The cannulas were anchored, using quick drying dental cement (Duralon®, Premier Dental Products, Norristown, PA, USA) on the flat skull surface previously scraped clean of periosteum. The stereotaxic coordinates used were based on the atlas of Slotnick and Leonard [22] and were as follows (in mm; from bregma): AP−6.4, LM±0.8; DV−1.0. The other details were as previously reported [5,8].

2.4. Drugs

Artificial cerebrospinal fluid (aCSF) was the vehicle used to prepare solutions of adenosine drugs and the antisense to CB_1 receptor. The following drugs were used in the present investigation: CHA, 5′-*N*-ethylcarboxamidoadenosine (NECA) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), (Research Biochemicals, Natick, MA, USA); Δ^9-THC; antisense oligodeoxynucleotide to CB1 receptor, its mismatch and PTX. The aCSF was employed in making solutions of the PTX, the antisense and the mismatch to CB_1 receptor whereas only an aliquot of 100% DMSO was used to aid in the dissolution of CHA, NECA, and DPCPX in aCSF. However, the vehicle used in the dissolution of Δ^9-THC was 100% DMSO.

2.5. Drug microinfusion technique

All drugs used in the present investigation were administered directly into the cerebellum except ethanol, which was given systemically (i.p.). All intracerebellar microinfusions were made into the same cerebellar cortical area, using the Hamilton microsyringes (25 μl) mounted on an infusion pump (Model 22, Harvard Apparatus, South Natick, MA, USA). The infusions of all drugs and oligodeoxynucleotides were made at a constant rate of 0.1 μl/min and were targeted specifically to the culmen of the anterior lobe of the cerebellum, which is regarded as 'spinocerebellum.' The targeted region of the cerebellum has a direct and indirect input from the spinal cord via mossy fibers as well as receiving cortico-pontocerebellar projection from the sensory motor cortex. The 1-μl and 100-nl infusion volumes of intracerebellar treatments of Δ^9-THC and other drugs (CHA, NECA, DPCPX, PTX), respectively, were found adequate in view of the consistent and reproducible response after their microinfusion. The guide cannulas, following their implantations, were plugged with stainless steel stylets to prevent clogging. Prior to actual microinfusions, the stylets were removed and using the fine-tipped dental broach the guide cannulas were cleaned. This was followed by lowering the injector cannula (30 gauge) through the guide cannula to the desired D-V coordinate. The length of the injector cannula, connected through PE-10 (Clay Adams) polyethylene tubing with Hamilton microsyringe, was such to allow a 1.0-mm protrusion beyond the tip of the guide cannula. Each microinfusion in the entire study was carried out for 60 s and the injector cannula was kept in the same position for an additional 60 s to ensure complete drug delivery during which time the animals were allowed to move freely. An air bubble separated the drug solution in the injector cannula from the water in PE-10 tubing and the microsyringe to help monitor the drug microinfusion.

2.6. Histology

The verification that the drug was microinfused to the target site was accomplished by administration of 100 nl of India Black ink via the same guide cannulas. Immediately after the infusion of stain, the animals were killed, their brains removed and fixed in 4% formalin and processed as previously reported [5]. The tissue sections were examined in order to determine the correct placement of the guide cannulas. Only the data from animals showing correct placement of guide cannulas based on histology and distribution of stain were included in the results. Based on previous work, less than 2% animals were rejected due to either surgery and or incorrect cannula placement. Little variation was noted within a single group and there was no significant overall variation noted between the groups. Tissue damage due to the placement of guide cannula and the extent of overall dispersion of drug microinfusions were comparable to what we have been previously reporting [5,8]. Dispersion studies (data not included) based on microinfusion of ^{3}H-labeled adenosine agonist (CHA) and antagonist (DPCPX) demonstrated that the diffusion of intracerebellarly microinfused drugs was always confined within the cerebellum.

2.7. Motor coordination

A mouse rotorod treadmill (UGO Basile, Verese, Italy)

calibrated for a fixed speed of 20 rpm was used to evaluate the motor coordination. The animals were always subjected to prescreening prior to their use in actual rotorod experiment, to rule out any inborn defect such as cerebellar and possible brain damage due to microsurgery. The prescreening also establishes the normal coordination in the animals based on our arbitrarily selected walking time of 180 s. Based on repeated observations during our previous studies, less than 3% of all mice prescreened failed to meet this test. Successfully prescreened mice ($n=5$) received intracerebellar microinfusion of Δ^9-THC or one of adenosine agonits/antagonist and/or followed within 5 min by 0.5 g–2 g/kg i.p. ethanol. However, the animals were subjected to rotorod evaluation every 15 min post-ethanol for 60 min. The animals that received no ethanol injection and were treated with Δ^9-THC microinfusion alone or additionally with drug(s) other than ethanol followed the rotorod protocol, which involved motor evaluations every 10 min post-Δ^9-THC microinfusion for 40 min. Control animals received intracerebellar microinfusion of the same volume of the vehicle (100% DMSO) and were evaluated by rotorod procedure in exactly the same manner as the treated group. Selection of the motor impairment dose of Δ^9-THC was based on a separate dose–response study the results of which are presented in Fig. 1. Other details of the rotorod procedure were as previously published [2–5,8,9]. The arbitrary criterion established in our laboratory for the normal motor coordination is the ability of the animals to walk on the rotorod treadmill for 180 s in three consecutive attempts. The changes in the motor coordination were expressed graphically as the time an animal walked on the rotorod treadmill at each evaluation time after intracerebellar microinfusion of the drug compared to pre-drug walking time.

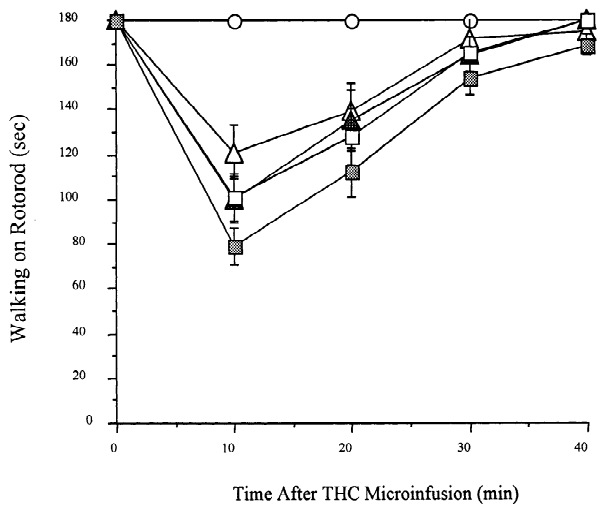

Fig. 1. Effect of various doses of Δ^9-THC on normal motor coordination following intracerebellar microinfusion. Each point represents the mean±SEM of eight mice. ○, Vehicle (100% DMSO) 1 μl; (△) Δ^9-THC 15 μg/1 μl; (▲) Δ^9-THC 20 μg/1 μl; (□) Δ^9-THC 25 μg/1 μl; (■) Δ^9-THC 30 μg/1 μl. Significant motor impairment was noted following 15-, 20- and 25-μg doses of Δ^9-THC at 10 and 20 min and after 30 μg at 10, 20 and 30 min post-microinfusion [ANOVA, followed by planned comparisons of the means yielded: F(1,20)=5.688 and 8.205, $P<0.05$; F(1,22)=10.621 and 25.757, $P<0.05$; F(1,22)=20.409 and 54.417, $P<0.05$; F(1,26)=5.038–96.482, $P<0.05$, respectively].

2.8. Statistical analysis

The motor coordination data from rotorod experiments were subjected to statistical analyses using analysis of variance (ANOVA) with repeated measures in order to test the significance of interaction between drug treatment and evaluation time periods. This was followed by one-way ANOVA and Newman–Keuls post-hoc analysis at each time period to determine the significance of any difference in the treatment groups. The CRUNCH statistical package version 3.0 (Crunch Software, Oakland, CA, USA) was used to perform the rotorod data analysis. A $P<0.05$ was taken as a level of significance.

3. Results

Selection of the motor impairing dose (15 μg) of Δ^9-THC was based on a separate dose–response study the results of which are presented in Fig. 1. The motor impairment observed following the intracerebellar microinfusion of Δ^9-THC (15, 20, 25 and 30 μg) into the spinocerebellar area in the present investigation was quick and roughly dose-dependent (Fig. 1). The effect lasted less than 50 min post-Δ^9-THC microinfusion as the animals nearly recovered their normal motor coordination after 40 min of microinfusion (Fig. 1). The peak effect was noted within 10 min post-microinfusion, which was not associated with any visible sedation or loss of righting reflex. The vehicle (100% DMSO; 1 μl) did not alter the normal motor coordination (data not shown). There was a significant interaction between various Δ^9-THC treatments and the time periods ($F_{12,204}=7.741$; $P<0.05$). The motor impairment was marked at 10 and 20 min post-microinfusion periods. A significant treatment–time interaction was observed at 15 μg dose ($F_{3,60}=6.573$; $P<0.05$). The motor impairment was significant at 10 and 20 min post-microinfusion periods but not at 30 and 40 min. Similarly, a significant time vs. treatment interaction was observed following microinfusion of 20 and 25 μg dose (Fig. 1) ($F_{3,66}=22.862$, $P<0.05$ and $F_{3,72}=39.429$, $P<0.05$, respectively). The motor impairment following 20 and 25 μg doses was also significant only at 10 and 20 min post-microinfusion evaluations. The highest (30 μg) dose was the only Δ^9-THC treated group in which significant motor impairment was observed at three evaluation periods, i.e., 10, 20 and 30 min post-microinfusion. Significant time and

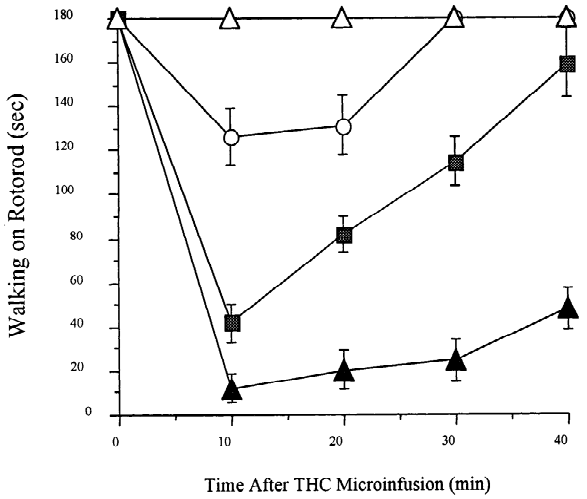

Fig. 2. Effect of intracerebellar pretreatment with A_1-selective agonist, CHA, antagonist, DPCPX (100 ng) and non-selective $A_1=A_2$ agonist, NECA, on intracerebellar microinfusion of Δ^9-THC (15 μg)-induced motor incoordination. Each point represents the mean±SEM of six mice. ○, Δ^9-THC; △, DPCPX+Δ^9-THC; ▲, CHA 4 ng+Δ^9-THC; ■, NECA 6 ng+Δ^9-THC. Significant accentuation by CHA and NECA of Δ^9-THC-induced motor impairment was observed at all four evaluation time periods [ANOVA, followed by planned comparisons of the means yielded: F(1,20)=19.871–78.587, $P<0.05$ and F(1,16)=4.601–9.644, $P<0.05$, respectively].

drug treatment interaction at this dose was noted ($F_{3,78}=40.354$, $P<0.05$).

Fig. 2 shows the effect of intracerebellar microinfusion of CHA, NECA and DPCPX on intracerebellar Δ^9-THC-induced motor incoordination. The selection of CHA, NECA and DPCPX dose was based on a separate dose–response study (data not included) as well as our previously published work [6,8]. The behavioral interaction between CHA and Δ^9-THC was marked with a significant ($F_{3,60}=21.126$, $P<0.05$) time and drug treatment interaction. The accentuation by CHA of Δ^9-THC-induced motor incoordination was significant at all four (10, 20, 30 and 40 min) evaluation time periods. Similar marked accentuation of Δ^9-THC-induced motor incoordination by intracerebellar NECA microinfusion was noted at all four (10, 20, 30 and 40 min) evaluation time periods. There was a significant drug treatment and time interaction ($F_{3,48}=17.75$; $P<0.05$). The Δ^9-THC-induced motor incoordination was virtually abolished by intracerebellar pretreatment with A_1-selective antagonist DPCPX associated with a significant ($F_{3,33}=11.095$; $P<0.05$) time–drug treatment interaction.

Figs. 3 and 4 show behavioral interactions between ethanol and Δ^9-THC. A dose-related accentuation of Δ^9-THC-induced motor incoordination by ethanol was observed (Fig. 3). Also, the sub-threshold ataxic doses of Δ^9-THC exhibited marked motor incoordination in presence of a motor incoordinating dose (2 g/kg, i.p.) of ethanol (Fig. 4). A significant motor incoordination was observed when microinfusion of 15 μg dose of Δ^9-THC

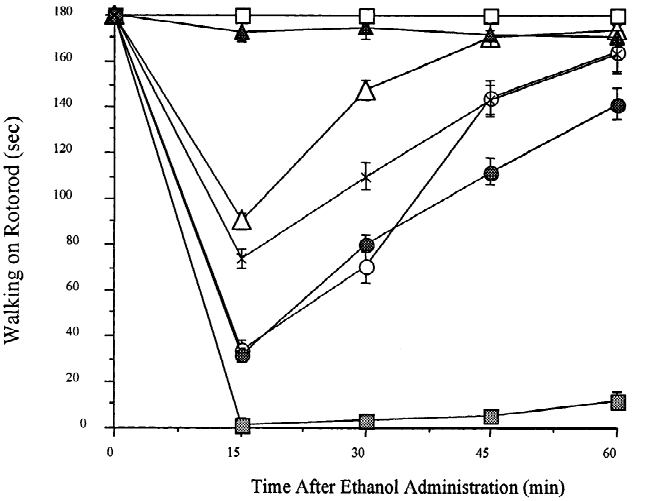

Fig. 3. Behavioral interactions between intracerebellar Δ^9-THC (15 μg) and various doses (0.5, 1.0 and 2.0 g/kg, i.p.) of ethanol on normal motor coordination in mice. Each point represents mean±SEM of eight mice. ○, EtOH 2 g; △, EtOH 1.0 g; ▲, EtOH 0.5 g; □, saline i.p.; ■, Δ^9-THC+EtOH 2 g; ●, Δ^9-THC+EtOH 1 g; ×, Δ^9-THC+EtOH 0.5 g.

was followed by 0.5 or 1.0 g/kg i.p. dose of ethanol. These doses of ethanol when administered alone produced no or little effect on normal motor coordination (Fig. 3). Similarly, virtually no change in the normal motor coordination was observed following the intracerebellar microinfusion of sub-threshold ataxic doses, 100, 500 and 1000 ng, of Δ^9-THC (data not shown). However, in the presence of a motor incoordinating dose, 2 g/kg, i.p., of ethanol, marked motor incoordination was observed following the microinfusion of these three sub-threshold ataxic doses of

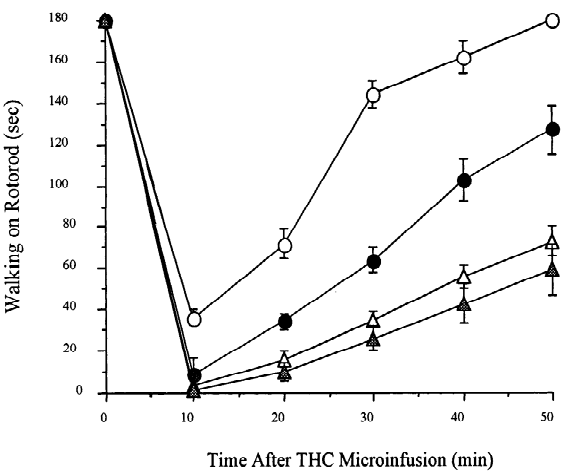

Fig. 4. Behavioral interactions between ethanol, 2 g/kg i.p., and various subthreshold intracerebellar doses (100, 500 and 1000 ng) of Δ^9-THC on normal motor coordination in mice. Each point represents the mean±SEM of eight mice. ○, vehicle (100% DMSO) 1 μl+EtOH; ●, Δ^9-THC 100 ng+EtOH; △, Δ^9-THC 500 ng+EtOH; ▲, Δ^9-THC 1000 ng+EtOH. Significant increase in Δ^9-THC-induced motor impairment was observed after ethanol at all four time periods [ANOVA, followed by planned comparisons of the means yielded: F(1,9)=324.55–5565.91, $P<0.05$.

Δ^9-THC (Fig. 4). There was a significant time–treatment interaction ($F_{3,27}=27.355$, $P<0.05$) in Δ^9-THC 500 ng+ ethanol (2 g/kg; i.p.) group (Fig. 4). The motor incoordination was significant at all four evaluation time periods (10, 20, 30 and 40 min).

Pretreatment with antisense oligodeoxynucleotide to CB_1 receptor, but not its mismatch, virtually abolished the motor incoordination produced by Δ^9-THC (Fig. 5). However, no attenuation in Δ^9-THC-induced motor incoordination was observed in the animals pretreated with the mismatch to CB1 (Fig. 5). The time and drug treatment interaction was marked in the antisense pretreated animals compared to Δ^9-THC alone ($F_{3,66}=19.320$, $P<0.05$). There was no effect of mismatch on Δ^9-THC-induced motor incoordination and the response curves were nearly superimposable (Fig. 5).

Fig. 6 shows the effect of intracerebellar pretreatment of antisense and mismatch to CB_1 on the accentuation by CHA of Δ^9-THC-induced motor impairment. A marked decrease in the accentuation by CHA of Δ^9-THC-induced motor impairment was observed in animals pretreated with antisense but no alteration of Δ^9-THC-induced motor incoordination was noted after pretreatment with the mismatch. A significant time–treatment interaction between the CHA+Δ^9-THC groups with and without the antisense pretreatment was noted ($F_{3,45}=14.810$, $P<0.05$). The attenuating effect of antisense pretreatment on CHA+ Δ^9-THC-induced motor incoordination was significant at all four evaluation time periods. Again as in Fig. 5, the lack of an effect of mismatch pretreatment on CHA+Δ^9-THC-induced motor incoordination was evident from the

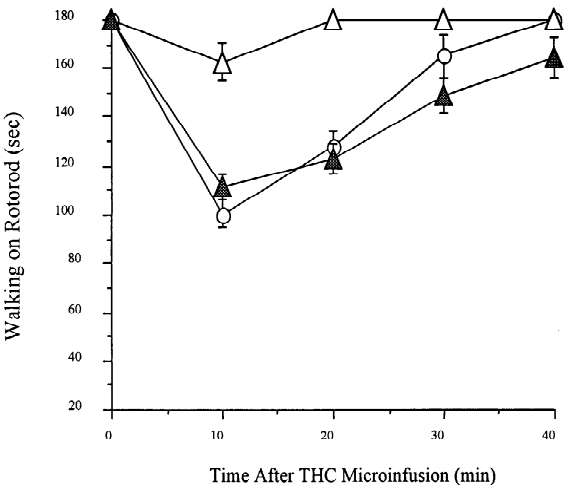

Fig. 5. Effect of intracerebellar pretreatment with antisense oligodeoxynucleotide and its mismatch to CB1 receptor (3 μg/12 h; total 72 h) on intracerebellar Δ^9-THC (25 μg)-induced motor incoordination in mice. Each point represents mean±SEM of six mice. ○, Δ^9-THC 25 μg; △, antisense (pretreatment)+Δ^9-THC 25 μg; ▲, mismatch (pretreatment)+ Δ^9-THC 25 μg. Significant attenuation in Δ^9-THC-induced motor impairment by CB_1antisense treatment was observed at 10- and 20-min evaluation time periods [ANOVA, followed by planned comparisons of the means yielded: F(1,22)=23.720 and 15.764, $P<0.05$, respectively].

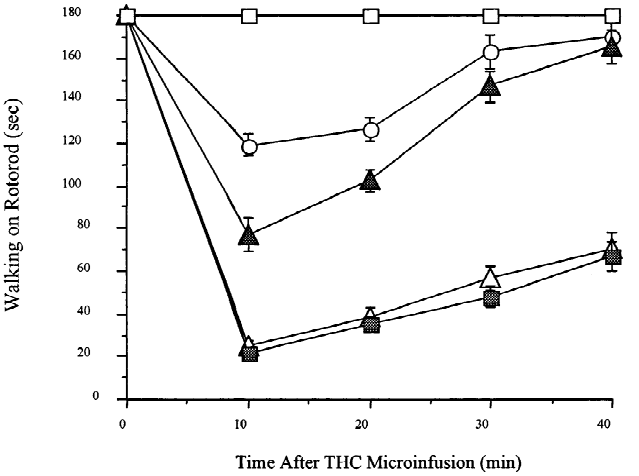

Fig. 6. Effect of intracerebellar pretreatment with antisense oligodeoxynucleotide and its mismatch to CB1 receptor (3 μg/12 h: total 72 h) on intracerebellar Δ^9-THC (15 μg)- and CHA (4 ng)+Δ^9-THC (15 μg)-induced motor incoordination in mice. Each point represents mean±SEM of six mice. ○, Δ^9-THC; △, CHA+Δ^9-THC; ■, mismatch (pretreatment)+CHA+Δ^9-THC; □, antisense (pretreatment)+Δ^9-THC; ▲, antisense (pretreatment)+CHA+Δ^9-THC. Significant attenuation by CB_1 antisense of CHA+Δ^9-THC-induced motor impairment was noted at all four evaluation time periods [ANOVA, followed by planned comparisons of the means yielded: F(1,15)=36.602–58.728, $P<0.05$].

virtual overlap of CHA+Δ^9-THC response curves in the mismatch pretreated and non-pretreated groups (Fig. 6). No significant time and pretreatment interaction was noted ($F_{3,33}=0.209$, $P<0.89$).

Fig. 7 shows the effect of intracerebellar pretreatment of pertussis toxin (PTX) on motor incoordination produced by Δ^9-THC and by CHA+Δ^9-THC. In the PTX-pretreated animals, the Δ^9-THC produced markedly reduced motor impairment compared to those which were not pretreated with PTX. There was a significant time–treatment interaction between the PTX-pretreated and the corresponding PTX-nonpretreated control group ($F_{3,75}=20.052$, $P<0.05$). The attenuation of Δ^9-THC-induced motor impairment due to PTX pretreatment was significant at 10, 20 and 30 min post-Δ^9-THC microinfusion evaluation periods. Similarly, in animals pretreated with PTX, the accentuation by CHA of Δ^9-THC-induced motor incoordination was markedly reduced compared to animals, which did not receive PTX pretreatment. A significant time–treatment interaction was observed ($F_{3,45}=7.386$, $P<0.05$). The interaction was marked at all four times. There was no change in the normal motor coordination following intracerebellar microinfusion of CHA and vehicle in PTX-pretreated animals (Fig. 7).

4. Discussion

The results of this investigation suggest that motor incoordination following direct intracerebellar microinfu-

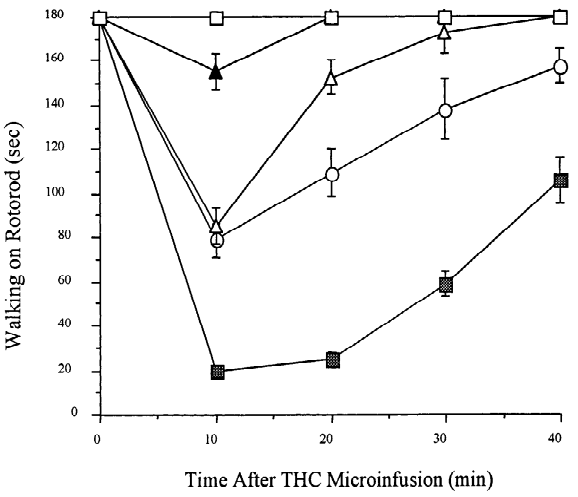

Fig. 7. Effect of intracerebellar pretreatment with pertussis toxin (PTX) (0.5 μg/500 nl) on intracerebellar Δ^9-THC (25 μg)- and CHA (4 ng)+Δ^9-THC (25 μg)-induced motor incoordination in mice. Each point represents mean±SEM of six mice. ○, Δ^9-THC; ▲, PTX (pretreatment)+Δ^9-THC; △, PTX (pretreatment)+CHA+Δ^9-THC; □, PTX (pretreatment)+CHA+vehicle (100% DMSO) 1 μl; ■, CHA+Δ^9-THC. Significant attenuation of Δ^9-THC- and CHA+Δ^9-THC-induced motor impairment was observed at 10, 20 and 30 min evaluation time periods [ANOVA, followed by planned comparisons of the means yielded: $F(1,25)=6.795$ to 22.747, $P<0.05$ and $F(1,15)=20.880$–200.125, $P<0.05$, respectively].

sion of Δ^9-THC is due to cerebellar CB_1 receptor participation. The results also indicate that Δ^9-THC-induced motor incoordination was modulated by cerebellar adenosine A_1 mechanism since intracerebellar microinfusion of both, A_1-selective agonist, CHA and nonselective $A_1=A_2$ agonist NECA, markedly enhanced the motor incoordination due to Δ^9-THC (Fig. 2) Unlike Δ^9-THC-treated animals which regained their normal motor coordination within 40–50 min, the recovery in animals pretreated with A_1-selective agonist was slower. The motor coordination was only 25% of the normal even at 40 min post-Δ^9-THC microinfusion. The enhancement of motor incoordination following intracerebellar microinfusion of nonselective $A_1=A_2$ agonist NECA, was also marked. However, the NECA-pretreated animals regained their normal motor coordination faster (i.e., within 40 min post-Δ^9-THC microinfusion) compared to CHA-pretreated group (Fig. 2). This points towards a predominant role for A_1-subtype in the adenosinergic modulation of Δ^9-THC-induced motor incoordination because of (i) a complete abolition of Δ^9-THC-induced motor incoordination by intracerebellar pretreatment with A_1-selective antagonist, DPCPX (Fig. 2) and (ii) relatively weaker accentuating effect of NECA (nonselective $A_1=A_2$ agonist; IC_{50} (nM): $A_1=11$, $A_2=16$) vs. robust accentuating effect by CHA (A_1-selective; IC_{50} (nM):$A_1=1$–2, $A_2=450$–1000), on Δ^9-THC-induced motor incoordination. The IC_{50} values of CHA and NECA for A_1, A_2 subtypes were obtained from RBI/Sigma Catalog of 1999. The observed cerebellar A_1-adenosinergic modulation of Δ^9-THC-induced motor incoordination is similar to the central adenosinergic modulation of ethanol-induced motor impairment which we have previously demonstrated [2–5,8,9].

The behavioral interaction between ethanol and Δ^9-THC observed in the present investigation was found to be significant and selective. There was a strong circumstantial evidence for such an interaction because: (i) brain adenosine has been shown by us to modulate ethanol-induced motor incoordination [2–5,8,9] and (ii) adenosine A_1 and CB_1 receptors are co-localized to axons and axonal terminals of granule cells in the cerebellar molecular layer [13,19]. The motor incoordination due to an ataxic dose (15 μg; intracerebellar; Fig. 1) of Δ^9-THC was so markedly enhanced in the presence of ethanol that the animals were virtually disabled. The animals practically could not walk on the rotorod treadmill up to 60 min post-ethanol injection (Fig. 3). Even the sub-threshold ataxic dose of 0.5 g/kg i.p. of ethanol, which alone produced little or no motor incoordination, markedly enhanced the motor incoordination due to the ataxic intracerebellar dose (15 μg) of Δ^9-THC (Fig. 3). This observation strongly suggested a selectivity of the interactions between the two psychoactive substances. The selectivity of the behavioral interaction was also suggested when each sub-threshold ataxic dose (100, 500 and 1000 ng) of Δ^9-THC failed to alter the normal motor coordination when microinfused alone but produced a marked and prolonged motor incoordination in presence of ethanol (2 g/kg; i.p.) (Fig. 4). The animals failed to regain their normal motor coordination even up to 60 min post-ethanol injection.

Once we observed that the cerebellum mediates the motor incoordinating effect of Δ^9-THC (Fig. 1) and the cerebellar A_1 adenosinergic mechanism modulates it (Fig. 2), we investigated if this effect of the cannabinoids was mediated by the cerebellar CB_1 receptor. This was indeed indicated by the data from the experiments with the antisense oligodeoxynucleotide and its mismatch to CB_1 receptor. Following the intracerebellar pretreatment with the antisense, the Δ^9-THC-induced motor incoordination was virtually abolished (Fig. 5). In contrast, the animals pretreated with the mismatch exhibited no change in the motor incoordination produced by the standard ataxic dose of Δ^9-THC (Fig. 5). In fact, the rotorod response curves of Δ^9-THC in the naïve and the mismatch pretreated animals were identical and overlapping (Fig. 5). The data from the antisense study, therefore, provided strong evidence to support that motor incoordination produced by intracerebellar Δ^9-THC is due to the participation of the cerebellar CB_1 receptor.

Further support to the adenosinergic modulation of Δ^9-THC-induced motor incoordination was provided by the results of the experiments in which the antisense pretreatment markedly attenuated the accenuating effect of CHA on Δ^9-THC-induced motor incoordination (Fig. 6). Again,

the mismatch pretreatment exhibited no effect on the CHA+Δ^9-THC response. The response curve in the mismatch pretreated animals was nearly identical to CHA+Δ^9-THC response curve in the naïve animals (Fig. 6). The marked enhancement by CHA of the Δ^9-THC-induced motor incoordination in the mismatch group further suggested that the cerebellar adenosine modulates Δ^9-THC-induced motor incoordination (Fig. 6). The mediation of Δ^9-THC-induced motor incoordination appeared to be via the CB_1 receptor as suggested by the antisense and its mismatch data (Fig. 5). The CB_1 is known to be a G-coupled receptor. To establish that cerebellar CB_1 is coupled to G_i proteins, the effect of intracerebellar pretreatment with PTX on Δ^9-THC-induced motor incoordination was also investigated. The results shown in Fig. 7, clearly indicated that CB_1 receptor is indeed coupled to PTX-sensitive G_I or G_o protein in the cerebellum. This was suggested because motor incoordinating effect of Δ^9-THC was virtually abolished due to PTX pretreatment (Fig. 7). The PTX-pretreatment also markedly antagonized the accentuation by CHA of Δ^9-THC-induced motor incoordination, indirectly supporting that the cerebellar A_1 adenosine receptor modulates the Δ^9-THC-induced motor incoordination (Fig. 2). It is well established that A_1 adenosine receptor is coupled to G_i/G_o protein.

The anatomical co-localization of adenosine A_1 and CB_1 receptors and their negative coupling to adenylate cyclase (AC) via PTX-sensitive G proteins within the cerebellum provide the basis of plausible explanation for the observed behavioral responses. The observed motor impairment by Δ^9-THC (Fig. 1) via CB_1 receptors (Fig. 5), its accentuation by adenosine A_1-selective agonist, CHA and non-selective $A_1=A_2$ agonist, NECA and its attenuation by A_1-selective antagonist, DPCPX (Fig. 2) could be due to alteration of AC and the consequent changes in the intracellular cAMP activity. We have proposed in our recent reports [5–7,18] that a decrease and increase in the intracellular cAMP activity may be functionally correlated with ethanol-induced motor impairment and reduction or abolition of motor impairment, respectively. Consequently, an activation of CB_1 receptors by Δ^9-THC would be expected to increase the activity of specific G_I protein and subsequently inhibit the activity of AC to which the G_I is coupled and reduce intracellular cAMP. The accentuation of Δ^9-THC-induced motor impairment by A_1 agonist CHA and non-selective $A_1=A_2$ agonist NECA, suggested modulation most likely via cerebellar A_1 adenosine receptor. These observations indicated that: (i) the further increase in Δ^9-THC's motor incoordination by CHA and NECA was most likely via participation of A_1 receptor and (ii) the antagonism by DPCPX of Δ^9-THC-induced motor incoordination involved interaction between A_1 and CB_1 receptors most probably at the post-receptor site of the AC–cAMP signal transduction pathway. The marked attenuating effect of PTX-pretreatment on Δ^9-THC- and CHA+Δ^9-THC-induced motor incoordination, could be due to uncoupling of A_1 and CB_1 receptors from their specific G_i proteins. Such an uncoupling of receptors could result in a failure of signal transduction along AC–cAMP pathway with ultimate abolition or marked decrease in the effector response (i.e., absence of enhanced motor incoordination due to Δ^9-THC and CHA+Δ^9-THC). The common decrease/abolition of Δ^9-THC-induced motor incoordination by DPCPX, antisense oligodeoxynucleotide to CB_1 receptor, or PTX most likely are due to different mechanisms. For example, the abolition of Δ^9-THC's motor incoordination effect by A_1-selective antagonist, DPCPX, was likely to be due to blockade of A_1 receptor thereby eliminating the adenosinergic modulation and accentuation of Δ^9-THC's motor incoordination effect. The DPCPX data, therefore, suggested: (i) an involvement of cerebellar adenosine in Δ^9-THC's motor incoordination effect; and (ii) failure of activation of A_1 receptors by the basal extracellular adenosine due to A_1 blockade by DPCPX. Therefore, there would be no signal generation from the A_1 receptors and a failure of signal transduction to AC–cAMP ultimately would produce no effector response. The abolition of Δ^9-THC-induced motor incoordination by antisense to CB_1 receptor, however, was due to 'knockdown' of the number and function of CB_1 receptor as a result of blockade of the translation of its mRNA. Consequent to antisense treatment, CB_1 mRNA will be interrupted causing reduction/abolition of CB_1 receptors. Therefore, Δ^9-THC would be able to produce no or little receptor stimulation due to absence or marked reduction in the CB_1 receptor density. Again, a failure in signal transduction to AC–cAMP pathway ultimately would result in no effector response. Finally, the marked attenuation of response by PTX treatment, as explained above, most likely was due to uncoupling of receptors. Thus, the data with DPCPX, PTX, and antisense oligodeoxynucleotide to CB_1 discussed above may suggest participation of a common final site along the AC–cAMP signaling pathway in the observed effector response, i.e., a decrease in Δ^9-THC-induced motor incoordination was observed in spite of pretreatment with diverse drugs (DPCPX, PTX, antisense to CB_1). Therefore, these collective results are consistent with a role of PTX-sensitive G protein, as well as with adenosinergic A_1 modulation of Δ^9-THC-induced motor incoordination via CB_1 receptors

In summary, the results of the present investigation indicate an important role for the cerebellar cortex in affecting motor incoordination due to direct intracerebellar microinfusion of Δ^9-THC, the main psychoactive constituent of marijuana. Further, the data lend support to the suggestion that the cerebellar CB_1 receptor, which is coupled to PTX-sensitive G_I/G_o protein, participated in the expression of Δ^9-THC-induced motor incoordination. The data also support the hypothesis that within the cerebellum, the adenosinergic modulation of Δ^9-THC-induced motor incoordination is most likely via the adenosine A_1 receptor in association with the PTX-sensitive

G_i/G_o protein perhaps through participation of a common catalytic unit of AC.

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